MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1. Soil

An alluvial soil from the experimental farm of the Central Rice Research Institute, Cuttack was used in various experiments. Soil was air dried and after breaking the clods sieved through a 2-mm mesh and stored in polyethylene bags at room temperature.

The physico-chemical properties of the soil presented in Table 2 were determined using following methods:

Soil pH was measured at $1:1.25$ soil to water ratio using Elico digital pH meter with calomel glass electrode assembly.

Soil organic carbon was determined by Walkey and Black method and organic matter was calculated by multiplying the organic carbon values with 1.72 (Jackson, 1967).

The soil was analysed for clay, silt and sand employing the Bouyoucos hydrometer method (Black, 1965).

The cation exchange capacity (CEC) of the soil was determined using normal ammonium acetate ($\text{pH } 7.0$) (Jackson, 1967). The total nitrogen content of the soil was estimated by Kjeldahl method (Jackson, 1967).
Table 2. Physico-chemical characteristics of the soil used

<table>
<thead>
<tr>
<th>Location</th>
<th>Soil</th>
<th>Total nitrogen (%)</th>
<th>CEC (me/100 g soil)</th>
<th>pH*</th>
<th>Organic matter (%)</th>
<th>pH**</th>
<th>Sand (%)</th>
<th>Silt (%)</th>
<th>Clay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRRI, Cuttack, Orissa</td>
<td>Alluvial</td>
<td>0.11</td>
<td></td>
<td>6.2</td>
<td>1.61</td>
<td>25.6</td>
<td>12.6</td>
<td>61.8</td>
<td></td>
</tr>
</tbody>
</table>

* Estimated by taking 1:1.25 soil-water slurry.

** Estimated by ammonium acetate (pH 7.0) method.

a Measured by taking 1:1.25 soil-water slurry.
b Estimated by Walkley-Black method.
c Estimated by ammonia acetate (pH 7.0) method.
d Bouyoucos hydrometer method.
e Estimated by Kjeldahl method.
3.2. Pesticides

Chemical configuration of pesticides used in this study are given in Figs. 1 and 3.

Technical formulations of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-isomers of hexachlorocyclohexane (HCH) 99.1% purity, used in studies on their degradation by rhizosphere and non-rhizosphere soil suspension and in bacterial cultures, were obtained from Lachat Chemicals, Mequon, Wisconsin, U.S.A.

Technical hexachlorobenzene (HCB), pentachlorobenzene (PCB) and pentachloronitrobenzene (PCNB) were gifted by Dr. K. Raghu, Bhabha Atomic Research Centre, Bombay.

The commercial dust formulation of HCH containing 50% active ingredient (a.i.), used for soil application in the pots and fields was obtained from M/s Das Enterprise, Calcutta.

Authentic standard of $\gamma$-PCH was gifted by Dr. N. Kurihara, Radioisotope Research Centre, Kyoto University, Kyoto, Japan.

3.3. Degradation of HCH isomers by suspension from HCH-treated and untreated soils

Experiments were conducted in the greenhouse and field to determine whether the phenomenon of accelerated (aerobically or anaerobically mediated) biodegradation of HCH can develop after repeated additions of HCH to planted (rice) or unplanted soils (flooded or nonflooded).
Figure 3. Chemical Configuration of Organochlorine Pesticides

**Hexachlorobenzene**

**Pentachlorobenzene**

**Pentachloronitrobenzene**
3.3.1. Preparation of suspension from unplanted or planted (to rice) soils (HCH-treated and untreated)

In the field experiment conducted in dry season (February-May), the commercial wettable-powder formulation of HCH was mixed with water and then applied as a spray to the unplanted or planted soils in field and greenhouse experiments. The field plots were 6 x 4 m in size. In the greenhouse experiments, earthenware pots (25.5 x 9.5 cm) containing 5 kg of soil were used. Flooded and nonflooded (60% water-holding capacity) conditions were maintained and HCH was applied at 1 kg a.i./ha to the plots and 10 ug/g to the pots. In both field and greenhouse experiments, the first application of HCH was made 10 days after flooding or moistening (nonflooded) the soil. A second application was made 15 days later (day 25) and a third application (in some experiments) on day 40. Fifteen days after every application (before the next application was made) of HCH, duplicate 1 g surface (1-2 cm) soil samples, collected from HCH-treated and untreated field plots or pots, were shaken with 10 ml sterile distilled water in presterilized test tubes (200 x 25 mm) to form the soil suspension used as inoculum for the γ-HCH degradation studies.

In the field experiment to determine the possible development of the phenomenon of accelerated degradation of γ-HCH after repeated additions of HCH to a flooded soil
planted to rice, rice seedlings (variety Vanaprabha; 25-day-old) were transplanted to puddled flooded plots (6 x 4 m). A commercial formulation of HCH mixed with water was sprayed at 1 kg a.i./ha on planted plots 25, 40 and 55 days after transplanting. Water, surface (1-2 cm) soil, and rhizosphere soil samples were collected 15 days after the first (before the second application) and the second application of HCH. To prepare rhizosphere soil suspension, rice seedlings were uprooted and the roots were carefully washed with water to remove large soil aggregates and then the soil closely adhering to the roots were shaken with 100 ml of sterile distilled water in 250 ml Erlenmeyer flasks for 1 h on a shaker and the resulting suspension was used as rhizosphere soil suspension.

Also, surface soil samples were collected from plots planted to rice after 17 days (3 days before harvest; moist soil with no standing water in the field) and 41 days (21 days after harvest when there was no standing water in the field and the soil had started to dry out) after third application of HCH. Suspensions of these surface soil samples from HCH-treated plots were prepared as described above.

The above field experiment on accelerated degradation of γ-HCH by HCH-acclimatized soil suspension was conducted in dry season (February to May). A repeat
experiment was conducted in the wet season (August to November) to determine the seasonal effects, if any, on the development of the factor causing accelerated degradation of \( \gamma \)-HCH. Soil, rhizosphere soil and water samples, collected 15 days after second application of HCH to flooded plots (nonflooded series could not be included due to the difficulty in controlling the moisture level in the rainy season) were tested for their ability to degrade \( \gamma \)-HCH, added to the mineral salts medium, under aerobic conditions as described for the dry season samples.

3.3.2. Degradation of \( \gamma \)-HCH, added to a mineral salts medium, by suspension from HCH treated and untreated soil

A mineral salts medium \([(NH_4)_2HPO_4, 0.5 \, g; \, MgSO_4 \cdot 7 \, H_2O, 0.2 \, g; \, FeSO_4 \cdot 7 \, H_2O, 0.001 \, g; \, K_2HPO_4, 0.1 \, g; \, Ca(NO_3)_2 \cdot 0.01 \, g; \, \text{distilled water, 1 litre; pH 7.0} \] was shaken with analytical grade \( \gamma \)-HCH for 48 h and then sterilized by filtration through a Millipore filter \((0.3 \, \mu m)\). Ten millilitre portions of this medium were dispensed in sterile 100 ml Erlenmeyer flasks and inoculated with 1 ml of soil suspension (flooded and nonflooded) from untreated or HCH-treated plots or pots, collected 15 days after first and second additions of HCH. The uninoculated medium served as control. The samples were incubated under intermittent
shaking (for 4 h after every 4 h) to provide aerobic conditions. At periodic intervals, 1 ml portions of the inoculated or uninoculated medium were withdrawn aseptically from duplicate flasks and shaken with 2 to 4 ml of hexane for 2 to 3 min for extraction of the HCH residues and the HCH residues in hexane fraction were analysed by gas-liquid chromatography.

3.3.3. Degradation of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-isomers of HCH by suspension of HCH-treated soil

In another experiment to test the aerobic degradation of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-isomers of HCH by HCH-enriched soil suspension, respective isomers were dissolved in acetone and 0.1 ml of acetone containing 50 $\mu$g of respective isomer was dispensed separately into presterilized 100 ml Erlenmeyer flask. After evaporation of acetone under room temperature, 10 ml portions of sterilized mineral salts medium were added to each flask and shaken for 24 h to provide a final concentration of 5 $\mu$g/ml of medium for each isomer. The mineral salts medium containing the individual HCH isomers was inoculated with 1 ml of soil suspension from flooded or nonflooded HCH treated pots (collected 15 days after the second application of HCH). Uninoculated medium served as control. At periodic intervals, contents in duplicate flasks each of uninoculated and inoculated medium for each
isomer were shaken with 10 to 30 ml of hexane for 30 min and residues of respective HCH isomers in hexane fraction were analysed by glc.

3.3.4. Degradation of \( \gamma \)-HCH in anaerobically versus aerobically incubated mineral salts medium by HCH-acclimatized soil suspension

Whether there is any enrichment of anaerobic \( \gamma \)-HCH-degrading principle after repeated additions of HCH to planted (rice) plots and unplanted pots, was tested as follows: \( \gamma \)-HCH was dissolved in acetone and 0.1 ml of \( \gamma \)-HCH was added to sterilized 100 ml Erlenmeyer flasks. After evaporation of acetone at room temperature, 10 ml aliquots of mineral salts medium supplemented with 0.1% yeast extract were added to each flask and shaken for 24 h to provide a final concentration of 7-8 \( \mu \text{g/ml} \) of medium. Yeast extract was added to the medium, because co-metabolism has been implicated in the anaerobic degradation of \( \gamma \)-HCH (Heritage and MacRae, 1977b; Ohisa and Yamaguchi, 1978; MacRae et al., 1984). After 24 h equilibration, the mineral salts medium supplemented with \( \gamma \)-HCH and 0.1% yeast extract was inoculated with 1 ml of suspension of surface plus sub-surface (upto 5 cm depth) soil from HCH-treated pots or 1 ml of soil suspension (rhizosphere and non-rhizosphere) from HCH-treated flooded plots planted to rice (after 10 days of second application of HCH). A non-rhizosphere soil suspension was prepared by shaking 1 g of
soil from unplanted flooded plots with 10 ml of sterile distilled water in presterilized tubes (200 x 25 mm). One set of inoculated and uninoculated medium was transferred aseptically into separate presterilized tubes (100 x 15 mm) and incubated under anaerobic conditions as described under Section 3.7. Simultaneously another set in 100 ml Erlenmeyer flasks was incubated in a shaker to obtain aerobic conditions at room temperature (28 ± 2°C). Samples inoculated with soil suspensions from untreated pots or plots and incubated under anaerobic or aerobic conditions served as controls. Samples were withdrawn from duplicate flasks or tubes at periodic intervals and analysed for γ-HCH after extraction with hexane.

3.3.5. Accelerated degradation of γ-HCH by soils collected at different depths from HCH-treated flooded rice field

Soil samples collected at different depths from the HCH-treated field were tested for an increase in the rate of γ-HCH degradation. There was no standing water in the field 14 days after the third application of HCH and a vertical trench was dug into the HCH-treated plot to a depth of 50 cm. Moist soil samples were collected carefully from soil depths of 1-2 cm, 10-12 cm, 30-32 cm and 45-47 cm by inserting a sterile spatula into the wall of the trench. Suspensions of these soil samples (1 g) from different depths were prepared in sterile distilled water (10 ml).
3.3.6. Persistence of the factor causing accelerated degradation of $\gamma$-HCH in HCH-treated soil (flooded or nonflooded)

The persistence of the factor causing accelerated degradation of $\gamma$-HCH was examined in a soil suspension collected from HCH-treated pots. After 15 days of second application of HCH (November, 1990) surface soil samples (flooded or nonflooded) were collected at monthly intervals till February, 1992. Mineral salts medium (10 ml) containing $\gamma$-HCH (6 to 7 $\mu$g/ml) was inoculated with 1 ml of soil suspension from untreated and HCH-treated pots (flooded or nonflooded) and incubated under aerobic conditions. Uninoculated medium served as control. At regular intervals, 1 to 2 ml samples were withdrawn aseptically from duplicate flasks and analysed for $\gamma$-HCH after extraction with hexane.

3.3.7. Degradation of $\gamma$-HCH by soil suspension collected from HCH-treated rice fields before and after harvest

To determine the presence of the $\gamma$-HCH degradation factor in HCH-treated rice field before and after harvest,
an experiment was conducted in dry season (February to May). Surface soil samples were collected after 17 days (3 days before harvest; moist soil with no standing water in the field) and 41 days (21 days after harvest when there was no standing water in the field and the soil had started to dry out) after third application of HCH. Mineral salts medium (10 ml) containing \( \gamma \)-HCH (5 to 6 \( \mu \)g/ml) was inoculated with 1 ml of this soil suspension and incubated under aerobic conditions. Uninoculated medium served as control. At periodic intervals, 1 ml portion of the inoculated or uninoculated medium was withdrawn aseptically from duplicate flasks and shaken with adequate hexane for 2 to 3 min for extraction of \( \gamma \)-HCH residues and subsequent analysis by gas-liquid chromatography.

3.3.8. Effect of temperature on the accelerated aerobically mediated degradation of \( \gamma \)-HCH by HCH-enriched soil suspension

To study the effect of temperature on the accelerated aerobic degradation of \( \gamma \)-HCH by HCH enriched soil suspension (flooded or nonflooded), the mineral salts medium (10 ml) supplemented with \( \gamma \)-HCH (6 to 7 \( \mu \)g/ml) was inoculated with 1 ml of the flooded or nonflooded soil suspension, collected 15 days after the second addition of HCH. Uninoculated medium served as control. Duplicate flasks of uninoculated and inoculated medium were incubated at 20, 25, 30 and 35°C in B.O.D. incubators. At periodic
intervals, 1 to 2 ml samples were withdrawn from each of the duplicate flasks and analysed for γ-HCH residues after extraction with hexane by glc.

3.3.9. Effect of moisture-temperature interaction on the persistence of the factor causing accelerated degradation of γ-HCH in HCH-treated soil

In a follow-up study, moisture soil samples (20 g) were collected from HCH-treated pot after second application of HCH. Soil samples were incubated in Petri dishes at 20, 25 and 35°C in B.O.D. incubators. Loss of water from a separate set for each temperature was replenished daily while in another set, no water was added to compensate for the loss by evaporation during incubation at respective temperatures. Amount of water present originally in 20 g of moist soil used for incubation was estimated by keeping 20 g of moist soil in Petri dishes at a higher temperature (80°C). Also, loss of water by evaporation in 5 days at different temperatures (20, 25 and 35°C) was evaluated by subtracting the weight of soil after 5 days at respective temperatures from the original weight at the start.

At regular intervals, 1 g portion of the soil was removed from both sets (replenished and unreplenished at each temperature (20, 25 and 35°C) and soil suspension was prepared in 10-ml of sterile distilled water. Portions (10 ml) of the mineral salts medium supplemented with γ-HCH, was inoculated
with 1 ml soil suspension from the respective sets for each temperature and incubated under aerobic conditions at room temperature (28 ± 2°C). Uninoculated medium served as control. At periodic intervals, 1 to 2 ml portions of the inoculated and uninoculated medium were withdrawn aseptically from each of duplicate sets and γ-HCH remaining in the medium was estimated by glc after extraction with hexane.

3.3.10. Accelerated aerobic degradation of γ-HCH by soil suspension from HCH-treated rice-straw amended soil (flooded or nonflooded)

One of the practices used in rice culture is to apply organic sources such as rice straw to increase the fertility of the soil. The aim of this experiment was to determine whether the phenomenon of accelerated degradation of γ-HCH can develop in flooded or nonflooded soils amended with rice straw after repeated additions of HCH. The soil (5 kg) was thoroughly mixed with rice-straw at 0.5% (w/w) in pots (25.5 x 9.5 cm). After 10 days of flooding or moistening (nonflooded) commercial formulation of HCH was added to the soils (rice straw amended or unamended) at a concentration of 10 µg/g soil. Fifteen days after second addition of HCH, 1 g of surface soil samples were collected from each of two replicate pots and shaken with 10 ml of sterile distilled water. The mineral medium (10 ml) containing γ-HCH (6 to 7 µg/ml) was inoculated with 1 ml
of this soil suspension and incubated under aerobic conditions. Uninoculated medium served as control. At periodic intervals, 1 to 2 ml samples were withdrawn aseptically from each flask and analysed for \( \gamma \)-HCH by glc after extraction with hexane.

3.4. **Microbial involvement in the accelerated degradation of HCH isomers by suspension from HCH-treated soils**

Experiments were conducted to determine the role of microorganisms in the accelerated degradation of \( \gamma \)-HCH by HCH-acclimatized soil suspension.

3.4.1. **Degradation of \( \gamma \)-HCH by sterilized versus non-sterilized soil suspensions of HCH-treated soil (flooded or nonflooded)**

Microbial role in the accelerated degradation of \( \gamma \)-HCH by suspension of HCH-treated soil (flooded or nonflooded) was ascertained as follows: Twenty millilitre portions of soil suspension of surface (1-2 cm) from HCH-treated flooded and nonflooded pots (collected 15 days after the second application of HCH) were sterilized by autoclaving at 121°C for 30 min. Mineral salts medium (10 ml) supplemented with \( \gamma \)-HCH (8 to 9 \( \mu \)g/ml) was inoculated with 1 ml of sterilized and non-sterilized soil suspension (flooded or nonflooded) and incubated under room temperature. Uninoculated mineral salts medium served as control. At periodic intervals, 1 ml sample from each of duplicate flask
was withdrawn aseptically and analysed for $\gamma$-HCH by glc after extraction with hexane.

3.4.2. Populations of $\gamma$-HCH degrading microorganisms after successive applications of HCH to flooded and non-flooded soils

The population density of $\gamma$-HCH degrading microorganisms (aerobic and anaerobic) was estimated after successive applications of HCH to flooded and nonflooded soils by using most-probable-number (MPN) method. In this greenhouse experiment, three repeated additions of HCH were made to unplanted soil contained in pots at 15-day intervals. After each successive addition of HCH, 1 ml soil suspension (flooded or nonflooded) was serially diluted up to $10^{-10}$ and 0.3 ml of each dilution of soil suspension was added to 2.7 ml of mineral salts medium supplemented with $\gamma$-HCH (6 to 7 $\mu$g/ml) in presterilized test tubes (150 x 15 mm). Five replicates of the medium inoculated with each dilution (upto $10^{-10}$) were incubated under aerobic conditions at room temperature ($28 \pm 2^\circ$C). Uninoculated medium served as control. After 10 days of incubation under aerobic conditions $\gamma$-HCH residues were extracted from 5 replicates of each dilution by shaking 3 ml of the sample with hexane and analysed by glc.

To estimate the population of anaerobic $\gamma$-HCH degrading microorganisms in HCH-treated soil, after each of the three successive additions of HCH 1 ml of the serially
diluted (up to $10^{-10}$) soil suspension was inoculated with 10 ml portions (5 replicates) of mineral salts medium containing $\gamma$-HCH and 0.1% yeast extract in presterilized test tubes (100 x 15 mm) and incubated in a glass chamber under anaerobic conditions as described under section 3.7. Uninoculated media served as control. After 30 days of incubation, 1 ml portion of the sample was withdrawn aseptically from 5 replicates of each dilution (up to $10^{-10}$) and residues of $\gamma$-HCH were extracted with hexane and analysed by glc. The populations of aerobic and anaerobic $\gamma$-HCH degrading microbes were estimated by using most probable number assay of Alexander (1982).

3.4.3. Isolation of HCH-degrading bacteria from suspension of HCH-treated soils

For more convincing evidence for the microbial role in the accelerated aerobic degradation of $\gamma$-HCH by suspension from HCH-retreated flooded field, attempts were made to isolate HCH degrading bacteria and then determine their ability to degrade $\gamma$-HCH in a mineral salts medium under aerobic conditions.

For isolation of HCH-degrading bacterium, 10 ml portion of mineral salts medium containing $\gamma$-HCH (6 to 7 µg/ml) was inoculated with 1 ml soil suspension from HCH-retreated flooded field and then incubated under aerobic conditions at room temperature ($28 \pm 2^\circ C$). After complete
disappearance of \( \gamma \)-HCH from the inoculated medium in about 5 to 10 days, 1 ml of this medium was again added to 10 ml of mineral salts medium containing \( \gamma \)-HCH (6 to 7 \( \mu \)g/ml) and incubated for 5 days. This was repeated 5 times at 5-day intervals for selective enrichment of \( \gamma \)-HCH degrading bacteria. After 5th transfer, 1 ml of the inoculated medium was serially diluted up to \( 10^{-10} \), and sterile mineral salts medium containing \( \gamma \)-HCH (6 to 7 \( \mu \)g/ml) was inoculated with 1 ml of each dilution. Uninoculated medium served as control. Flasks were incubated at room temperature (28 ± 2°C). After 5 days, 1 to 2 ml samples were withdrawn aseptically from each flask for each dilution and analysed for \( \gamma \)-HCH by glc after extraction with hexane.

The maximum dilution from which \( \gamma \)-HCH disappeared completely in 5 to 10 days was plated on the same mineral salts medium but containing glucose (1%), peptone (0.1%), agar (1.8%) and 3 to 4 \( \mu \)g of \( \gamma \)-HCH per ml of medium. Three distinct types of bacterial colonies appeared on the agar plate within 5 days of incubation at room temperature (28±2°C). Mineral salts medium (10 ml) containing \( \gamma \)-HCH (as a sole carbon source) was inoculated with individual bacterial colonies belonging to the three types that appeared on the agar plate to test for their ability to degrade \( \gamma \)-HCH. Only one of the three distinct bacterial colony types was selected. This bacterium was further purified by several transfers on mineral salts agar medium
supplemented with 1% glucose, 0.1% peptone and 3 to 4 µg of γ-HCH per ml of medium. This bacterium was identified as *Sphingomonas paucimobilis* based on its morphological, physiological and biochemical characteristics.

### 3.4.4. Aerobic degradation of γ-HCH as a sole source of carbon by *Sphingomonas paucimobilis* as related to its proliferation

In an effort to determine the ability of the bacterium, *Sphingomonas paucimobilis* to utilise γ-HCH as a sole carbon source for its growth and degradation, 10 ml portions of the sterilized mineral salts medium containing γ-HCH (6 to 7 µg/ml) were dispensed into 100 ml Erlenmeyer flasks and inoculated 0.1 ml suspension of the bacterium (22 x 10³ cells) in sterile distilled water. Uninoculated medium served as control. Both inoculated and uninoculated medium were incubated under room temperature. At periodic intervals, 1 to 2 ml of the medium were withdrawn from triplicate flasks and γ-HCH residues were extracted with hexane and analysed by glc. The cell proliferation of the bacterium in the presence of γ-HCH as a sole source of carbon was studied as follows: Serial dilutions (10⁻¹ to 10⁻⁵) of the inoculated medium were prepared and 0.1 ml of each dilution was simultaneously plated on the same mineral salts medium supplemented with 1% glucose, 0.1% peptone, 1.8% agar and 3 to 4 µg/ml of γ-HCH. Colony forming units (CFU) appearing on the agar plates were counted after 3 or 4 days.
3.4.5. Aerobic degradation of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-isomers of HCH by \textit{Sphingomonas paucimobilis}

\textit{Sphingomonas paucimobilis}, isolated by serial dilution technique from HCH-enriched flooded soil, was tested for its ability to degrade $\alpha$-, $\beta$-, $\gamma$- and $\delta$-isomers of HCH. About 5 $\mu$g/ml of each isomer dissolved in 0.1 ml acetone was added to separate 100 ml Erlenmeyer flasks. After evaporation of acetone, 10 ml of mineral salts medium was equilibrated for 24 h on an orbital shaker. A suspension of \textit{Sphingomonas paucimobilis} was prepared in 5 ml sterile distilled water from 48 h old culture of the bacterium grown on the mineral salts medium supplemented with 1% glucose, 0.1% peptone and 3 to 4 $\mu$g/ml of $\gamma$-HCH. The sterile mineral salts medium containing respective HCH isomers was inoculated with 0.1 ml (61 x $10^2$ cells) of bacterial suspension and incubated under intermittent shaking to provide aerobic conditions. At periodic intervals 10 ml of the samples were shaken with 10 to 40 ml of hexane for 30 min and the respective HCH isomers in hexane extract were analysed by glc.

3.4.6. Growth curve of $\gamma$-HCH degrading bacterium \textit{Sphingomonas paucimobilis} in a mineral salts medium supplemented with 1% glucose and 0.1% peptone

To determine the growth curve of \textit{Sphingomonas paucimobilis}, 15 ml portions of the mineral salts medium supplemented with glucose and peptone were dispensed into
100-ml side-arm Erlenmeyer flasks and inoculated with *S. paucimobilis*. Triplicate flasks were inoculated with 0.1 ml of the bacterial suspension containing $85 \times 10^2$ cells. Uninoculated medium served as control. At regular intervals, bacterial growth was determined by reading the absorbance of uninoculated and inoculated medium in a Spectronic-20 (Bausch and Lomb) at 610 nm wave length.

In a follow-up experiment to determine the effect, if any, of the HCH isomers on the cell size of *S. paucimobilis*, the bacterium was grown in the above medium [mineral salts medium + glucose (1%) + peptone (0.1%)] without and with $\alpha$-, $\beta$- and $\gamma$-HCH (0, 2 and 10 µg/ml). After 24 h of incubation, a loopful of the bacterial suspension was smeared on a glass slide. After the gram-staining the cell size (length) of the bacterium was measured under the microscope (10 x 100 magnification).

3.4.7. Degradation of $\beta$-HCH by *Sphingomonas paucimobilis* in the presence or absence of HCH-enriched soil suspension (sterile or nonsterile)

In another experiment, degradation of $\beta$-HCH by *Sphingomonas paucimobilis* in the presence or absence of sterilized HCH enriched soil suspension was determined.

About 5 to 6 µg of analytical grade $\beta$-HCH dissolved in 0.1 ml of acetone was added to presterilized 100 ml Erlenmeyer flasks. After evaporation of acetone, 10 ml portions of mineral salts medium were added to each flask
and then shaken on an overhead shaker for 24 h. The medium containing $\beta$-HCH was inoculated with 1 ml of the sterile and nonsterile HCH-enriched soil suspension (collected 15 days after second addition of HCH) and 0.1 ml of the suspension of *Sphingomonas paucimobilis* (42 x $10^3$ cells) in sterile distilled water. Uninoculated medium served as control. Both inoculated and uninoculated medium were kept under intermittent shaking to provide aerobic conditions at room temperature (28 ± 2°C). At periodic intervals, $\beta$-HCH residues remaining in the medium from duplicate flasks were extracted with 10 to 40 ml of hexane for 30 min and analysed by glc.

3.4.8. Effect of rice-straw on the degradation of $\beta$-HCH by *Sphingomonas paucimobilis* in nonflooded and flooded soil

The effect of rice straw on the degradation of soil applied $\beta$-HCH by *Sphingomonas paucimobilis* was examined. For flooded conditions, 20 g portions of the soil were placed in 250 ml Erlenmeyer flasks and flooded with 25 ml of sterile distilled water. For nonflooded conditions, 20 g portions of the soil placed in 250 ml Erlenmeyer flasks, were moistened with 3.6 ml of sterile distilled water. The rice straw at 0.25% (w/w) was added to the soil and thoroughly mixed with the soil prior to the addition of water for flooded or moist conditions (nonflooded). After 10 days of flooding or moistening under laboratory conditions, $\beta$-HCH
was added to the soil in 0.1 ml of acetone to make a final concentration of 5 to 6 μg/g of soil. For inoculation of the soil with *Sphingomonas paucimobilis*, the soils (unamended and rice straw-amended) were inoculated with 1 ml of bacterial suspension prepared in sterile distilled water from 48 h culture of *Sphingomonas paucimobilis*. Uninoculated flooded or nonflooded soils served as control. Flooded soil samples were incubated under intermittent shaking at room temperature (28 ± 2°C). At regular intervals, residues of \( ^{14} \text{HCH} \) were extracted from the soil as described under Section 3.5.2. and estimated by glc.

### 3.4.9. Aerobic degradation of \( \gamma \)-HCH and hexachlorobenzene (HCB), pentachlorobenzene (PCB), and pentachloronitrobenzene (PCNB) by *Sphingomonas paucimobilis*

In another experiment, the ability of HCH-degrading bacterium *Sphingomonas paucimobilis* to degrade hexachlorobenzene (HCB, fungicide), pentachlorobenzene (PCB), pentachloronitrobenzene (PCNB, fungicide), and \( \gamma \)-HCH was examined. These compounds dissolved in 0.1 ml of acetone or ethanol were added to separate 100 ml Erlenmeyer flasks. After complete evaporation of acetone or ethanol, 10 ml portions of mineral salts medium were dispensed into the flasks and equilibrated for 24 h. A suspension of *Sphingomonas paucimobilis* was prepared in sterile distilled water from 48 h old culture of the bacterium. The sterile mineral salts medium containing HCB, PCB, PCNB, and \( \gamma \)-HCH were separately inoculated with 0.1 ml of bacterial suspension. Uninoculated
medium served as control. The inoculated samples were shaken intermittently to provide aerobic conditions. Uninoculated and inoculated samples were incubated at room temperature (28 ± 2°C). At periodic intervals, samples were withdrawn from duplicate flasks and shaken with 10 to 20 ml of hexane for 20 to 30 min, and a pinch of Na₂SO₄ was added. Hexane fraction was analysed for HCB, PCNB, PCB and γ-HCH by glc.

3.4.10. Effect of heavy metals on the degradation of γ-HCH by *Sphingomonas paucimobilis*

The effect of heavy metals on the aerobic degradation of γ-HCH by *Sphingomonas paucimobilis* was examined. Different concentrations of aqueous solutions of heavy metals viz., Zn as zinc sulphate, Cd as cadmium chloride, Ag as silver nitrate and Hg as mercuric chloride were prepared and added to the γ-HCH supplemented mineral salts medium to provide the final concentrations ranging from 2 to 100 ppm. Medium supplemented with respective heavy metals and γ-HCH was sterilized by filtration through a Millipore filter (0.3 μm). Portions (10 ml) of this medium containing heavy metal and γ-HCH (for each heavy metal) was inoculated with 0.1 ml of the suspension in sterile distilled water of *Sphingomonas paucimobilis* obtained from a 48 h old culture, grown on mineral salts medium supplemented with 1% glucose, 0.1% peptone and 4 μg of γ-HCH. Uninoculated medium served as control. At periodic
intervals, 1 to 2 ml samples from the inoculated and unincubated medium were withdrawn aseptically from duplicate flasks and analysed for γ-HCH by GLC after its extraction in 2 to 3 ml of hexane. Analysis of the medium by atomic absorption spectroscopy showed that heavy metals, Cd and Zn were in solution at the concentrations used.

3.5. Extraction and analysis of HCH isomers

3.5.1. Extraction of HCH isomers from liquid medium

The residues of HCH isomers remaining in both unincubated and inoculated medium were extracted with hexane. Portions (1 to 2 ml) of liquid samples were withdrawn aseptically from each flask and then shaken with 1 to 6 ml of hexane and 50 mg of sodium sulphate for 5 min. When HCH isomers were applied to the medium at concentrations above their solubility range HCH residues remaining in the medium were extracted by shaking the medium in the whole flask with hexane (medium : hexane of 1:1 or 1:2) for 20 min in a rotary shaker. Likewise in one experiment, hexachlorobenzene, pentachlorobenzene and pentachloronitrobenzene were extracted from the whole flask.

3.5.2. Extraction of β-HCH residues from the soil

The method followed for extraction of β-HCH residues from the soil (rice straw-amended and unamended) was the
same as described earlier (Raghu and MacRae, 1966) with slight modifications. For extraction of $\beta$-HCH from flooded and nonflooded soil (Section 3.4.8), 25-ml of distilled water were added to nonflooded soil (to bring the same soil-water ratio as in flooded soil). The soil samples from each flask were transferred to 500 ml volumetric flasks with 100 ml of acetone and shaken for 1 h on an orbital shaker. After 1 h of equilibration with acetone, the contents were shaken with 20 ml of hexane for 1 h and 2% of sodium sulphate solution was added to each flask to make the volume up to 500 ml. $\beta$-HCH residues in the hexane fraction were quantified by glc after suitable dilution with hexane.

3.6. Gas-liquid chromatography (glc)

HCH isomers, extracted from liquid medium or soil in hexane, were analysed in a Perkin-Elmer gas chromatograph model 3920, equipped with a Ni$^{63}$ detector and a glass column (0.625 cm outside diameter; 2 m length) packed with 5% QF-1 on Chromosorb W, 60/80 mesh. Column, injector and detector temperatures were maintained at 190, 210 and 250°C, respectively, with a flow rate of carrier gas (95% argon + 5% methane) at 13 ml/min. Under these conditions, the retention time was 2.0 min for $\alpha$-HCH, 2.8 min for $\beta$-HCH, 2.42 min for $\gamma$-HCH, 3.42 min for $\delta$-HCH, 1.5 min for $\alpha$-PCH, 0.67 min for $\gamma$-PCH and 4.0 min for bacterial metabolite of HCH, 2.10 min for HCB, 1.42 min for PCB and 2.42 min for PCNB.
The HCH isomers and other pesticides were quantified using a standard calibration curve 0.2 to 1 ng for $\alpha$-HCH, 0.5 to 4 ng for $\beta$-HCH, 0.25 to 2 ng for $\gamma$-HCH, 0.5 to 4 ng for $\delta$-HCH, 0.5 to 2 ng for HCB, 0.2 to 1.2 ng for PCB and 1 to 4 ng for PCNB. The recovery of the four parent isomers of HCH, HCB, PCB and PCNB by the extraction and analytical procedures used in this study immediately after application to the mineral salts medium ranged from 95 to 100%.

3.7. Anaerobic system

Samples containing $\gamma$-HCH were incubated under anaerobic conditions of BBL Gas Pak™ system (BBL Microbiology Systems, Becton. Dickinson and Co., Cockeysville, MD., U.S.A.) as described earlier (Brahmaprakash et al., 1985). Anaerobic conditions were created by adding 10 ml of distilled water to a Gas Pak™ disposable $H_2 + CO_2$ generator envelope containing sodium borohydrate, sodium bicarbonate and citric acid. $H_2 + CO_2$ evolved provided anaerobic conditions in the chamber. A Gas Pak™ anaerobic indicator (fructose, 1.8%; potassium phosphate, 1.53%; sodium hydroxide, 0.35% and methylene blue, 0.025%) was placed in the anaerobic chamber as an indicator for anaerobiosis. The indicator remained white during incubation period confirming anaerobic conditions in the system. At periodic intervals, residues in duplicate samples were extracted with hexane and analysed for $\gamma$-HCH by glc after extraction in hexane and old Gas Pak™ envelopes were exchanged with the new ones.